Antibodies against GD2 Ganglioside Can Eradicate Syngeneic Cancer Micrometastases[1]

Helen Zhang, Shengle Zhang, Nai-Kong V. Cheung, Govindaswami Ragupathi, and Phillp O. Livingston[2]

Departments of Medicine [H. Z., S. Z., G. R., P. O. L.] and Pediatrics [N-K. V. C.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021

ABSTRACT

After 10 years of clinical trials in patients with advanced cancer, monoclonal antibodies (mAbs) against cell surface antigens have not lived up to their initial promise. One such cell surface antigen is the ganglioside GD2. GD2 is richly expressed at the cell surfaces of human neuroblastomas, sarcomas, and melanomas. We have described a murine lymphoma (EL4) that is syngeneic in C57BL/6 mice and expresses GD2, a mAb against GD2 (mAb 3F8), and we have prepared a conjugate vaccine (GD2-keyhole limpet hemocyanin plus immunological adjuvant QS-21) that consistently induces antibodies against GD2. We demonstrate here, for the first time in a syngeneic murine model, that passively administered and vaccine-induced antiganglioside antibodies prevent outgrowth of micrometastases, and we use this model to establish some of the parameters of this protection. The level of protection was proportional to antibody titer. Treatment regimens resulting in the highest titer antibodies induced the most protection, and protection was demonstrated even when immunization was initiated after tumor challenge. Treatment with 3F8 1, 2, or 4 days after i.v. tumor challenge was highly protective, but waiting until 7 or 10 days after challenge resulted in minimal protection. The results were similar whether number of liver metastases or survival was used as the end point. These results suggest that unmodified mAbs or antibody-inducing vaccines against GD2 (and possibly other cancer cell surface antigens) should be used exclusively in the adjuvant setting, where circulating tumor cells and micrometastases are the primary targets.

INTRODUCTION

Most mAb[3] treatments have been performed on patients with advanced disease, and the treatments were of short duration, with response of measurable disease as the end point. Responses have been rare. Occasional regression of measurable neuroblastoma, melanoma, and breast cancer lesions and more frequent regression of B-cell lymphomas have resulted in patients treated with mAbs against cell surface antigens, including: gangliosides GM2[4] (1), GD2 (2-5), and GD3 (6-8); HER2 neu (9); and lymphoma idiotype antigens (10, 11). Trials with mAbs against GD2 are a case in point. The response rate in children with GD2-positive cancers (primarily neuroblastomas) treated with mAb 14.G2a or 3F8 is between 0 and 25% (12, 13), and in melanoma patients treated with mAb 3F8, 14.G2a, or chimeric 14.18, the response rate is between 0 and 22% (13, 14). A chimeric 14.18-interleukin 2 fusion protein shown to be potent in a scid/scid xenograft model (15) is now being considered for clinical trials. Neither immunogenic GD2 vaccines nor a syngeneic animal model has been previously available, making it difficult to compare these various approaches or to test the many variables associated with antibody-mediated therapies in the setting of a normal immune system.

As opposed to the minimal benefit seen with mAbs in patients with advanced disease, there is an expanding body of evidence indicating that antibodies can protect against subsequent tumor challenge in experimental animals and prevent tumor recurrence in humans. mAbs against several protein or glycoprotein tumor antigens have resulted in significant protection from syngeneic tumors in the mouse (16-19), mAb R24 against GD3 has resulted in protection from syngeneic melanoma growth in hamsters (20), and mAbs against GD2/GD3 (21) or GD2 (22) have resulted in protection against human tumor challenges in nude mice. There is also evidence in humans that natural antibodies, passively administered antibodies, or vaccine-induced antibodies against cancer antigens can result in prolonged disease-free and overall survival in the adjuvant setting. (a) Paraneoplastic syndromes have been associated with high titers of natural (not induced by vaccine or passive administration) antibodies against onconeural antigens expressed on neurones and certain malignant cells. The antibodies are apparently induced by tumor growth and have been associated with autoimmune neurological disorders and, in addition, with delayed tumor progression and prolonged survival (23-25). (b) Patients with American Joint Commission On Cancer stage III melanoma and natural antibodies against GM2 ganglioside studied at two different medical centers have an 80-90% 5-year survival, compared to the expected 40% rate (26, 27). (c) Patients with small cell lung cancer and natural antibodies against small cell lung cancer had prolonged survival, compared to antibody-negative patients (28). (d) Patients with Dukes' C colon cancer treated with mAb 17-1A in the adjuvant setting had a significantly prolonged disease-free and overall survival, compared to randomized controls (29). (e) Antibody responses induced by vaccines in the adjuvant setting have been correlated with subsequent prolonged disease-free and overall survival (26, 27, 30-33).

Given the potential clinical importance of a variety of cell surface antigens, including ganglioside GD2, as targets for mAbs and cancer vaccines inducing an antibody response, we have identified a suitable syngeneic mouse model to address some of the variables associated with antibody-mediated protection from and therapy of cancer. EL4 is a lymphoma syngeneic in C57BL/6 mice that we have previously reported to express GD2 (34). It is a unique model, in that GD2 is also a human tumor antigen, against which there is not only a clinically active mAb but also a consistently immunogenic conjugate vaccine, GD2-KLH plus QS21. We demonstrate here that passively administered and vaccine-induced antibodies are able to prevent establishment of subsequently administered EL4 challenge and to eliminate EL4 micrometastases when administered after EL4 challenge, and we define some of the parameters of this protection.

MATERIALS AND METHODS

mAbs and Vaccine

The origins of mAb 3F8 (IgG3) against GD2 (35), mAb 696 (IgM) against GM2 (36), mAb 013 against a primitive human neuroectodermal bone tumor (37), and mAb 1E3 against Tn antigen (38) have been described. Neither 013

Table 1 *Experiments 1: liver metastases after i.v. challenge with EL4 lymphoma incubated with mAbs 3F8 (against GD2) and 696 (against GM2)*

mAb	No. of mice	No. of tumors in liver	Liver mass (g)
PBS (control)	8	57.8 ± 67.2	2.59 ± 1.03
mAb 696	5	94 ∓ 103	2.52 ± 1.21
mAb 3F8	5	0	1.23 ± 0.04[b]
mAb 696 + mAb 3F8	5	0	1.20 ± 0.14[b]

[a] After incubation with 100 μg/ml 3F8 and 50 μg/ml 696 for 1 h, 3 × 10⁴ EL-4 cells per mouse were injected (i.v.) into C57BL/6 mice. Thirty-four days after challenge, mice were sacrificed, and the livers were evaluated. Results are expressed as mean ± SD.

[b] *P* < 0.01, compared with PBS control group.

nor 1E3 reacts with EL4. Immunological Adjuvant QS-21, a purified saponin fraction (39), was obtained from Aquila Biopharmaceuticals Inc. (Worcester, MA). GD2 and GD2 conjugated to KLH were provided by Progenics Pharmaceuticals Inc. (Tarrytown, NY). Conjugation of GD2 to KLH was achieved by conversion of the GD2 ceramide double bond to aldehyde by ozonolysis and attachment to KLH by reductive amination in the presence of cyanoborohydride, as described previously for GD3 (40). Each GD2-KLH vaccine contained 10 μg of GD2 conjugated to 60 μg of KLH, plus 10 μg QS-21. Vaccines were administered s.c. three times at 1-week intervals, except in the final experiment, when they were administered at 4-day intervals.

Mice and Cell Lines

C57BL/6 mice (6 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The EL4 cell line was established from lymphoma induced in a C57BL/6 mouse by 9,10-dimethyl-1,2-benzanthracene. It has recently been shown to express GD2 ganglioside (34). EL4 was maintained in 10% FCS-RPMI. For tumor cell challenges, EL4 cells were washed three times in PBS, and 3 × 10⁴ cells (in the final experiment, 5 × 10³ cells) were injected i.v. into the tail vein. At the indicated time points, mice were sacrificed, and livers were removed, weighed, and fixed in 10% formalin. Metastases were also frequently present in lymph nodes and other sites (although rarely in the lungs), but hepatic metastases were easiest to quantitate. Hepatic metastases were detected as white nodules on the liver surface.

Serological Assays

ELISA. ELISAs were performed as described previously (41). GD2 or GM2 in ethanol was coated on ELISA plates at 0.1 μg/well. A series of antiserum dilutions were incubated with the coated ganglioside for 1 h. Secondary antibodies were alkaline phosphatase-conjugated goat antimouse IgG or IgM at a dilution of 1:200 (Southern Biotechnology Associates, Inc., Birmingham, AL). ELISA titer is defined as the highest dilution yielding an absorbance of 0.1 or greater over that of normal control mouse sera. mAbs 3F8 and 696 were used as positive controls in each assay.

Flow Cytometry. EL4 cells (3 × 10⁵) were incubated with 40 μl of 1:30 diluted antisera or 1:2 diluted mAb supernatant for 30 min on ice. After washing with 3% FCS in PBS, the cells were incubated with 20 μl of 1:15 diluted FITC-labeled goat antimouse IgG or IgM (Southern Biotechnology Associates, Inc.). The positive population of the stained cells was quantitated by flow cytometry (EPICS-Profile II; Coulter Co., Hialeah, FL), as described previously (41).

CDC. In 100 μl of 5% FCS in RPMI, 2 × 10⁵ EL4 cells were incubated with 10 μl of 1:10 mouse antiserum or 10 μg/ml mAb for 10 min. Thirty μl with 10 μl of 1:10 mouse antiserum or 10 μg/ml mAb for 10 min. Thirty μl of complement (guinea pig; Sigma Chemical Co.) were added and incubated at 37°C for 4 h. Thirty μl of 0.4% trypan blue were added, and after 3 min, dead and viable cells were counted (41).

Statistical Methods

Experimental groups were compared to controls for number of hepatic metastases, survival, or antibody titers using the Mann-Whitney two-sample *t* test (42).

RESULTS

Having previously shown that mAb 3F8 was able to bind to EL4 and induce potent CDC and antibody-dependent cell-mediated cyto-

toxicity (3, 13, 35), we performed a series of experiments progressively testing the ability f passively administered and then actively induced antibodies against GD2 to eradicate hepatic micrometastases (experiments 1 and 2) and to prolong survival (experiments 3–7).

Effect of mAb Administration on Hepatic Metastases (Experiments 1 and 2). In experiment 1, we mixed 3F8 or negative control mAb 696 with the EL4 lymphoma cells prior to challenge to confirm in vivo impact of antibody binding. EL4 cells were incubated for 1 h in vivo impact of antibody binding. EL4 cells were incubated for 1 h with PBS, mAb 696 (against GM2, which is minimally expressed on EL4), mAb 3F8, or mAbs 696 and 3F8 prior to i.v. challenge. All mice were sacrificed on day 34, hepatic metastases were counted, and livers were weighed (Table 1). Only EL4 preincubation with 3F8 ± 696 eliminated metastases. In experiment 2, mice were injected i.v. with PBS, negative control antibody 1E3 (100 μg), or one of three doses of 3F8 (50, 100, or 250 μg) 2 h before i.v. challenge with untreated EL4 cells. Mice were sacrificed at day 30. Administration of all three doses of 3F8 eliminated metastases in most mice (Table 2).

Effect of mAb Administration or Vaccination on Survival (Experiments 3–6). In experiment 3, two groups of six mice received a single i.v. injection of 200 μg of 3F8 1 day before or 2 days after EL4 i.v. challenge. Three additional groups of six mice were vaccinated i.v. challenge. Three additional groups of six mice were vaccinated three times (on days −21, −14, and −7) prior to EL4 challenge. They were vaccinated with PBS, 10 μg of GD2 mixed with 60 μg of KLH plus QS21 (negative controls), or 10 μg of GD2 conjugated to 60 μg plus QS21 (negative controls), or 10 μg of GD2 conjugated to 60 μg of KLH plus QS21. Mice receiving the conjugate vaccine survived significantly longer than did the control mice (*P* < 0.008), and one mouse was sacrificed on day 100 with no evidence of tumor. Five of six mice receiving 3F8 1 day before challenge and five of six mice receiving 3F8 2 days after challenge also remained tumor free (Fig. 1, *Experiment 3*). All negative control mice died by day 28.

Experiments 4 and 5 focused on treatment with mAb. In experiment 4, groups of four or five mice received PBS or 3F8 2 days or 4 days after EL4 challenge i.v. All 3F8-treated mice survived longer than did control mice (*P* < 0.004), and three mice in the 3F8 groups remained tumor free (Fig. 1, *Experiment 4*). Experiment 5 compared treatment with PBS or mAb O13 (negative controls) and treatment with 50 or 200 μg of 3F8, all administered 2 days after EL4 challenge i.v. (Fig. 1, *Experiment 5*). Once again, all 3F8-treated mice survived longer than did any control mouse (*P* < 0.004), and most mice (8 of 12) treated with either dose of 3F8 remained tumor free.

Experiment 6 again compared immunization prior to tumor challenge with mAb treatment at various intervals after challenge. All vaccinated mice again survived longer than did any control mouse (*P* < 0.004), and four of six mice remained disease free (Fig. 1, *Experiment 6b*). Most mice receiving 70 μg of 3F8 2 or 4 days after challenge remained disease free. However, the same dose 7 or 10 days after challenge had no significant effect (Fig. 1, *Experiment 6a*). Experiment 6 was a single experiment but is presented in two panels for greater clarity. Once again, the relevant negative control treatments (mAb R24 against GD3, which is not expressed on EL4, and

Table 2 *Experiment 2: liver metastases after i.v. injection of mAbs followed by i.v. EL4 challenge*

Treatment	No. of mice	No. tumors in liver	Liver mass (g)
PBS (control)	7	29.2 ± 14.8	1.90 ± 0.47
mAb 1E3 (100 μg/mouse)	9	17.6 ± 15.9	1.95 ± 0.72
mAb 3F8 (50 μg/mouse)	6	0[d]	1.03 ± 0.13
mAb 3F8 (100 μg/mouse)	6	4.3 ± 7.0[c]	1.17 ± 0.41
mAb 3F8 (250 μg/mouse)	6	0[d]	0.90 ± 0.16

[a] Challenge was with 3 × 10⁴ EL-4 cells 2 h after mAb injection. The mice were sacrificed 30 days after challenge, and the livers were evaluated. Results are expressed as mean ± SE.

[b] *P* < 0.01, compared with PBS control group.

[c] *P* < 0.02, compared with PBS control group.

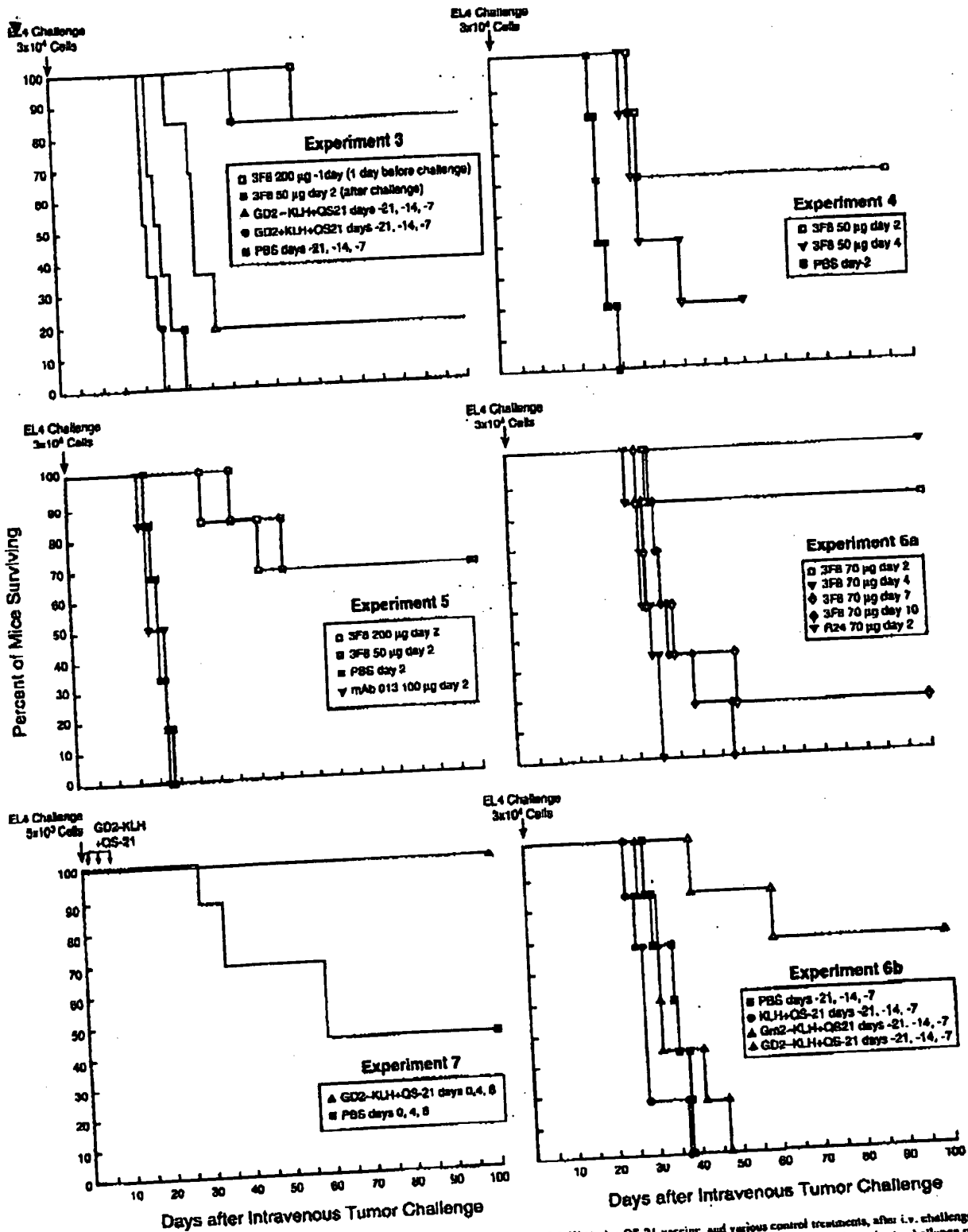[d] *P* < 0.001, compared with PBS control group.

Fig. 1. Survival of groups of four to six mice treated in five separate experiments with 3F8 mAb, GD2-KLH plus QS-21 vaccine, and various control treatments, after i.v. challenge with syngeneic EL4 lymphoma cells. 3F8 mAb against GD2 administered prior to challenge or 1-4 days after challenge and GM2-KLH plus QS-21 vaccination prior to challenge or starting immediately after challenge were both protective.

Table 3 *Antibody reactivity in sera of mice treated with GD2-KLH vaccine or mAb 3F8*

Treatment	No. of mice	Reciprocal ELISA titer		Flow cytometry (% positive cells)		CDC (% dead cells)
		IgM	IgG	IgM	IgG	
Experiment 3						
PBS (100 μl)	6	0/0	0/0	3.1-3.3/3.2	3.1-3.4/3.2	10-10/10
GD2 + KLH + QS21 (10 μg + 60 μg + 10 μg)	6	0-80/40	0	5.0-8.9/6.5	4.2-5.5/4.5	Not tested
GD2-KLH + QS21	6	40-640/80	80-2,560/640	18-88/57	22-93.4/60.5	20-60/40
3F8 (250 μg), 1 day before challenge	6	0/0	2,560-5,120/5,120	Not tested	99-99.3/99	90-95/95
Experiment 6						
PBS (100 μl)	6	0/0	0/0	1.5-2.9/2.0	1.4-2.6/1.7	10-15/10
KLH + QS21 (60 μg + 10 μg)	6	0/0	0/0	1.3-4.1/3.6	1.3-2.2/2.0	10-15/10
GD2-KLH + QS21	6	160-640/320	180-16,380/1,620	57-99/95	13-99/89	40-80/60
3F8 (250 μg)	6	0/0	1,620-4,860/3,240	Not tested	98-100/99	85-95/95

[a] Mice were bled 7 days after the third immunization with GD2 vaccine or 4-5 days after mAb 3F8 injection. Results are expressed as range/median.

vaccination with KLH plus QS21, GM2-KLH plus QS21, and PBS, which do not induce anti-GD2 antibodies) had no effect.

Correlation between Serum Antibody Titer and Survival. Serum anti-GD2 antibody titers immediately after 3F8 administration were not tested, but they ranged between 1:1620 and 1:4860 (median, 1:4860) 3-5 days later, except in experiment 4, in which they were between 1:180 and 1:4860 (median, 1:540). Vaccine-induced antibody titers ranged between 1:640 and 1:1620 for IgG and 1:80 and 1:1620 for IgM (Table 3). Comparable antibody titers by ELISA resulted in comparable reactivity by flow cytometry and complement-mediated cytotoxicity, whether due to 3F8 or vaccine administration. In both cases, protection from subsequent tumor challenge resulted. A correlation between antibody titer and in vivo protection is suggested by these results. Administration of 3F8 resulted in higher serum titers against GD2 than vaccine administration in both experiments ($P < 0.004$ for CDC) and greater protection ($P < 0.008$ for experiment 3). In experiment 4, in which 3F8 levels were lower than expected after 3F8 administration, survival was lower as well. Vaccine-induced antibody titers prior to challenge were higher in experiment 6 than in experiment 3, and protection was greater as well ($P < 0.025$).

Therapeutic Vaccination. Because 3F8 administration 7 or 10 days after EL4 challenge with 3×10^4 resulted in minimal protection, this suggested that vaccination after challenge, which was normally performed at weekly intervals and required 14-21 days for antibody induction, would be ineffectual. Consequently, we performed one final experiment aimed at testing the ability of vaccinations started after tumor challenge to prolong survival. In experiment 7, the number of EL4 cells per challenge was decreased from 3×10^4 to 5×10^3 cells, and the vaccines were administered on days 0, 3, and 7, beginning immediately after the challenge. Median IgM and IgG antibody titers on days 13 and 18 were both 1:320. Protection was again seen (Fig. 1, *Experiment 7*), although the difference was not statistically significant ($P = 0.15$).

DISCUSSION

The mechanism of antibody effect against bacteria is predominantly complement mediated inflammation and cytotoxicity (CDC: Ref. 43). Although other effector mechanisms have been suggested for GD2 antibody, such as inhibition of tumor cell substratum or extracellular matrix interactions (22), activation of immune effector mechanisms remains the most likely explanation. 3F8, the anti-GD2 mAb used here, is an IgG3 antibody that is particularly potent at inducing complement-mediated inflammation/cytotoxicity and antibody-dependent cell-mediated cytotoxicity. We have previously demonstrated, in melanoma patients, that natural or vaccine-induced IgM antibodies against GM2 ganglioside correlated with improved dis-

ease-free and overall survival (26, 44) and that a GM2-KLH plus QS21 vaccine induced IgM and IgG antibodies in melanoma patients, which were both able to mediate CDC (45). Fortuitously, the IgG subclasses were IgG1 and IgG3 (44-46), the two human subclasses best able to mediate CDC. The same applies to the murine model we describe here. IgM and IgG antibodies were induced in all vaccinated mice, these antibodies and administered 3F8 mAbs were able to mediate potent CDC, and antibody titers correlated with survival and inversely with the number of hepatic metastases. Although mAbs administered up until 4 days after challenge were able to completely prevent tumor growth in most mice, by 7-10 days after challenge, 3F8 administration had little effect. This strongly suggests that treatment with mAbs or vaccines inducing antibodies must be restricted to the adjuvant setting, where the targets are circulating tumor cells and micrometastases, and it may explain why mAb treatment trials in patients with measurable tumor burdens have not been more successful.

Passively administered and vaccine-induced antibodies were both able to protect against growth of micrometastases. There are advantages and disadvantages to each approach. Therapy in the adjuvant setting may require repeated treatments to maintain antibody titers over a prolonged period to overcome the issue of tumor cell dormancy and sanctuary sites. Except in immunosuppressed patients, this excludes murine mAbs, which would be eliminated within weeks by human antimouse antibodies. Chimeric, humanized, or human mAbs would overcome this issue but would be subject to elimination by anti-idiotypic antibodies. On the other hand, in the absence of human antimouse antibodies or anti-idiotypic antibodies, higher serum antibody levels than could be induced by vaccination are assured after mAb administration, and such antibodies have been or could be produced against most antigens. Vaccines against most defined tumor antigens are more practical to produce and administer because they can be administered s.c. and at longer intervals. Phase III trials with GM2-KLH and sialyl Tn-KLH vaccines that consistently induce moderate titers of antibodies against these antigens are currently ongoing in the adjuvant setting in patients with melanoma and breast cancer (33, 45). Because the antibody response seems to be polyclonal, antibody inactivation by anti-idiotypic antibodies has not been a problem and specific antibody levels have been maintained against GM2 by immunizations at 3- or 4-month intervals for over 2 years (45). However, even the most potent conjugate vaccines have not been able to induce consistent antibody responses against all antigens, and the titers are never as high as can be achieved with mAb administration. The results obtained here, demonstrating the ability of either approach to protect against tumor challenge and to eliminate micrometastases, in the absence of any detectable toxicity, argue strongly in favor of the careful use of either approach or the combination.

M2-KLH and GD2-KLH have both proven consistently immuno-
genic and safe in melanoma patients, whereas GD3 (the major mel-
anic and safe in melanoma patients, whereas GD3 (the major mel-
anoma ganglioside)-KLH has not proven so immunogenic (reviewed
in Ref. 47). Adjuvant therapy of melanoma might optimally include a
trivalent conjugate vaccine (GM2-KLH plus GD2-KLH), a humanized
anti-GD3 mAb, or a combination of bivalent vaccine plus mAb.

Vaccines against infectious diseases do not prevent infection; they
limit its spread from its point of contact. Postcontact boosts in anti-
body titers, even in protected hosts, attest to active infection at the
contact site. This is most striking when time has elapsed because the
original infection and antibody titers have fallen to low levels but rise
to protective levels within 4–7 days, preventing symptomatic infec-
tion. In patients with cancer, we see the adjuvant setting (after re-
moval of the primary cancer or positive lymph nodes) as being quite
similar to the picture in patients being reexposed to infectious dis-
eases. The primary targets in both cases are circulating pathogens and
microscopic spread, and in the case of infectious diseases, antibodies
are the primary method of protection. We demonstrate here, with
passively administered mAbs and vaccine-induced antibodies against
the defined cancer antigen GD2 ganglioside, that antibodies can also
protect mice against circulating syngeneic tumor cells and microme-
tastases. If antibodies of sufficient titer and potency to eliminate
circulating cancer cells and micrometastases could be maintained in
cancer patients as well, even metastatic cancer would have quite a
different implication. With continuing showers of metastases no lon-
ger possible, aggressive treatment of primary and metastatic sites
might result in long-term control.

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